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Expression of transient receptor potential mRNA isoforms and Ca^{2+} influx in differentiating human stem cells and platelets

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Abstract

Store-regulated Ca^{2+} entry (SOCE) is an important mechanism of elevating cytosolic $[Ca^{2+}]_i$ in platelets, though the Ca^{2+} influx channels involved are still unclear. We screened human platelets and their precursor cells (human stem cells and megakaryocytes) for the presence of candidate influx channels, i.e., isoforms of the Trp family of proteins. Primary stem cells were cultured with thrombopoietin to allow differentiation into megakaryocytes. The undifferentiated stem cells ($CD34^+$) showed mRNA expression of only a spliced variant Trp1A. Immature ($CD61^+/CD42b^{low}$) and mature ($CD61^+/CD42b^{high}$) megakaryocytes as well as platelets expressed in addition unspliced Trp1 as well as Trp4 (less abundant) and Trp6 isoforms. This unspliced isoform appeared to be specific for cells of the megakaryocyte/platelet lineage, since immature ($CD14^+/CD61^-/CD42b^-$) and mature monocytes expressed only the Trp1A isoform. This conclusion was confirmed by the presence of Trp1A, 3, 4 and 6 transcripts in the immature megakaryocytic Dami cell line, and of Trp1, 1A, 4 and 6 transcripts in the more mature CHRF-288 cell line. The up-regulation of Trp1, 4 and 6 in the lineage from primary stem cells to mature megakaryocytes and platelets was accompanied by increased influx of extracellular Ca^{2+} after pretreatment of the cells with thapsigargin or thrombin. Expression of new Trp isoforms in the differentiated cells is thus accompanied by increased SOCE.

Keywords: Calcium channel; Megakaryocyte; Monocyte; Stem cell; Trp; Platelet

1. Introduction

Almost all platelet activation processes, including shape change, secretion, thromboxane production and aggregation, depend on a threshold elevation in cytosolic $[Ca^{2+}]_i$. Many platelet agonists evoke a Ca^{2+} response that consists of both mobilisation of Ca^{2+} from intracellular stores, located in the endoplasmic reticulum, and Ca^{2+} influx from the extracellular medium [1–3]. The influx component poten-

Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium concentration; FITC, fluorescein isothiocyanate; $InsP_3$, inositol 1,4,5-trisphosphate; PCR, polymerase chain reaction; SOCE, store-operated Ca^{2+} entry; Trp, transient receptor potential

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tiates and extends the Ca^{2+} signal due to Ca^{2+} store release and, thereby, further stimulates the activation of platelets [4,5]. In platelets and their precursor cells, megakaryocytes, by far the most prominent mechanism of Ca^{2+} entry is that of store-operated Ca^{2+} entry (SOCE) [6–9], in which the influx is driven by depletion of intracellular Ca^{2+} stores [10,11]. Accordingly, Ca^{2+} influx is not only triggered by stimuli that cause generation of inositol 1,4,5-trisphosphate ($InsP_3$), but also by inhibitors of sarco/endoplasmic reticulum Ca^{2+}-ATPases such as thapsigargin [6–8,12–14]. Although patch-clamping studies with rat megakaryocytes have led to the demonstration of Ca^{2+}-release-activated currents (I_{crac}) [9,14], which are considered to originate from Ca^{2+} influx due to store depletion, the identity of the channels underlying SOCE in these and related cells is still elusive.

In recent years, the *Drosophila* transient receptor potential (Trp) protein is identified as a cation channel with properties compatible with SOCE. More than seven mammalian *trp* genes, homologue to that of the *Drosophila* protein, have nowadays been cloned [15–17]. Expression studies of the Trp proteins in Chinese hamster ovary, human embryonic kidney and other cell systems strongly suggest that at least several of these genes encode for cation channels involved in Ca^{2+} entry [18–20]. Supporting evidence for involvement of these channels in SOCE comes from work showing that anti-sense mRNA of Trp reduces the Ca^{2+} entry evoked by $InsP_3$-mediated store depletion [17,21]. The mRNA of Trp1 and Trp6 appears to be present in many organs and cell lines [22–25], whereas that of Trp3 and Trp4 is less widely expressed [16,23]. While the Trp7 homologue is mainly detected in heart and lung, the transcripts of Trp2 (vomeronasal organ) and Trp5 (brain) have an even more restricted tissue distribution [26]. The *trp1* gene expresses at least two mRNA forms, encoding for Trp1 and the shorter variant Trp1A, either of which may represent store-regulated, non-selective cation channels [17,18,27]. When transfected in model cells, the Trp4 and 5 channels have an increased permeability for Ca^{2+} over monovalent cations like Na^+ [16,26]. The closely related Trp3, 6 and 7 proteins may consist of receptor-operated rather than store-regulated cation channels, which are non-selective for Ca^{2+} [19,20]. Recent work suggests that the latter channels are activated by diacylglycerol [26,28]. Insight into the functional activity of the various Trp proteins is, however, hampered by the possibility that they may assemble into both homomeric and heteromeric multimers with different channel properties [29].

A recent paper reports on the presence of Trp1 and Trp3 mRNA in the human megakaryocyte-related cell lines, MEG01, Dami and HEL, but also on the apparent absence in platelets [30]. Being interested in the origin of the high SOCE in platelets, we have re-evaluated and extended this work using primary, human stem cells differentiating towards mature megakaryocytes, and using freshly isolated platelets and related cell lines. We found that the Trp forms becoming expressed in maturating megakaryocytes and megakaryocytic cell lines differ from those in stem cells and (immature) monocytes. This difference was accompanied by a dissimilarity in amount of Ca^{2+} influx into the cells following store depletion with thapsigargin.

2. Materials and methods

2.1. Cell isolation and culturing

Citrated umbilical cord blood was collected from healthy newborns after informed consent of the mother, and was used within 48 h of delivery. Mononuclear blood cells were isolated by centrifugation on a layer of Ficoll Paque (Pharmacia Biotech, Uppsala, Sweden). Human $CD34^+$ (My^+) stem cells were isolated using a magnetic Mini-MACS sorting system and a CD34 isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), according to the manufacturer's instructions. The cells were cultured in vitro for 1–10 days in IMDM (Flow Laboratories, Irvine, UK), containing 10% (v/v) human AB plasma, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin (Gibco, Breda, the Netherlands) at 37°C in a humidified atmosphere with 5% (v/v) CO_2. Thrombopoietin and stem cell factor (both from Pepro Tech, Rocky Hills, NJ, USA) were added at concentrations of 20 and 50 ng/ml, respectively.

The primary stem cells were identified as $CD34^+$/$CD61^-$/$CD42b^-$. Culturing for 5 or 10 days resulted

in enrichment of two consecutive maturation phases: immature megakaryocytes defined as $CD61^+/CD42b^{low}$ and mature megakaryocytes defined as $CD61^+/CD42b^{high}$. The immature and mature megakaryocytes were selected from the cultures by fluorescence-activated cell sorting on a FACS-Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA), using fluorescein isothiocyanate (FITC)-labelled antibodies F803 against CD61 (glycoprotein IIIa, integrin ß$_3$) and F802 against CD42b (glycoprotein Ibα), respectively. These antibodies were obtained from Dako (Glostrup, Denmark). Immature monocytes, defined as $CD14^+$ (LPS receptor$^+$), were isolated from 5-day cultured stem cells by fluorescence-activated cell sorting using a phycoerythrin-conjugated anti-CD14 antibody from Becton Dickinson (Leu-M3).

Megakaryoblastic Dami cells, from human origin, were cultured in IMDM supplemented with 10% (v/v) horse serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin, as described before [31]. The Dami cells were a kind gift of Dr. R.I. Handin (Brigham and Women's Hospital, Boston, MA, USA). CHRF-288–11 cells, a gift of Dr. M. Lieberman (University of Cincinnati, OH, USA), were grown in Fischer's medium supplemented with 20% horse serum [32]. Mature monocytes were obtained by apheresis of blood from healthy volunteers, and purified by counterflow centrifugal elutriation, following elsewhere described procedures [33].

Platelets were isolated by differential centrifugation of freshly isolated human blood (three healthy volunteers, 100 ml/donor), basically as described elsewhere [34]. Pelleted platelets were resuspended and washed twice in HEPES buffer, pH 6.6 (136 mM NaCl, 10 mM glucose, 5 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$ and 0.05% (w/v) bovine serum albumin). Suspensions of washed platelets from various donors were combined and repeatedly centrifuged at $200\times g$ for 5 min to remove remaining leukocytes. Contamination of the final suspension with erythrocytes and leukocytes was determined from cytospins and by counting these cells by phase-contrast microscopic scanning, using an intensified charge-coupled device camera connected to a UNIX-driven computer system [30]. In a typical sample, numbers of erythrocytes and leukocytes were lower than 2 and 1 per 10^8 platelets, respectively.

2.2. cDNA preparation and multiplication

2.2.1. Preparation of cDNA

Approximately 1×10^5 stem cells or megakaryocytes, 1×10^6 Dami cells or monocytes, or 2×10^{10} platelets were washed once in phosphate buffer, pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4). The washed cells were lysed in lysis buffer, pH 8.0 (500 mM LiCl, 100 mM Tris, 10 mM EDTA, 5 mM dithiothreitol and 1% (w/v) lithium dodecylsulfate) [35]. The cell lysates were incubated with 50 μg paramagnetic Dynabeads, which were covalently coupled to oligo$(dT)_{25}$ primers, and mRNA was isolated following instructions of the manufacturer (Dynal, Oslo, Norway). The mRNA-containing beads were washed twice in 600 μl Tris buffer, pH 8.0 (150 mM LiCl, 10 mM Tris, 1 mM EDTA and 0.1% (w/v) lithium dodecyl sulfate), and then washed twice in 300 μl Tris buffer without lithium dodecyl sulfate. After another wash in RNAse-free reverse transcriptase buffer, pH 8.3 (50 mM Tris, 40 mM KCl and 6 mM $MgCl_2$), the mRNA-containing beads were incubated at 40°C for 1 h in the same buffer, but supplemented with 1 mM dithiothreitol, 1 mM dNTPs (Pharmacia, Uppsala, Sweden), 15 units RNAse inhibitor (Fermentas, Hilversum, the Netherlands) and 200 units M-MLV reverse transcriptase (Gibco BRL, Gaitherburg, MD, USA). A single cDNA strand was formed by polymerisation to the oligo$(dT)_{25}$ primer-bead complex. This solid phase cDNA was incubated in 200 μl Tris buffer, pH 7.9 (10 mM Tris and 1 mM EDTA) at 94°C for 2 min, washed twice with the same buffer, and stored until use.

2.2.2. Polymerase chain reaction (PCR)

The solid-phase cDNA was washed twice in 50 μl of Tris buffer, pH 8.3 (50 mM KCl, 10 mM Tris, 2 mM $MgCl_2$ and 0.001% (w/v) gelatine). PCR was performed in the same buffer, but supplemented with 200 μM dNTPs, 1 ng specific forward and reverse primers (Table 1) (Eurogentec, Seraing, Belgium), and 0.4 units Taq polymerase (SuperTaq, HT Biotechnology, Cambridge, UK). The mixture was heated at 94°C for 2 min, prior to three cycles of denaturation (94°C, 30 s), annealing (52–60°C, 30 s, see Table 1) and elongation (72°C, 60 s). Subsequently, the beads were magnetically removed, and

supernatants were carried through 30–35 cycles of PCR, which were finalised by a 10-min incubation at 72°C. To control for cDNA (mRNA) content, parallel PCRs were carried out using primers for GAPDH (Table 1). The removed solid-phase cDNA was incubated in Tris buffer(pH 7.9) at 94°C for 2 min, washed twice, and stored until further use. The PCR products were analysed by electrophoresis using a 2% (w/v) agarose gel.

2.3. Analysis of Trp cDNA

2.3.1. Nested PCR

To analyse Trp1 and Trp1A PCR products, 1F/2R primers (Table 1) were used to start another PCR reaction, after which 2F/1R primers served to perform a second series of PCR cycles. The reaction was carried out as described above. However, PCR cycles consisted of 30 s denaturation (94°C), 30 s annealing (54°C) and 60 s elongation (72°C), followed by a final step at 72°C for 5 min. PCR products were analysed on a 2% agarose gel. For confirmation, Trp1 and Trp1A PCR products were digested with *Pst*I (Gibco) at 37°C for 1 h, and restriction products were analysed on gel by comparison with Eurogentec smart ladder markers.

2.3.2. cDNA sequencing

Trp1, Trp1A and cDNA fragments were excised from gel and isolated by electro-elution [36]. Trp3 and 6 fragments were directly purified from gel using a PCR product purification kit supplied by Boehringer Mannheim (Germany). About 30 ng of the PCR fragments were sequenced using the fluorescent dideoxynucleotide terminator method from Applied Biosystems (Alameda, USA) [37]. Briefly, 30 ng of PCR product was added to 8 μl terminator-ready reaction mixture (Applied Biosystems), 30 ng primer and milli-Q water, at a total reaction volume of 20 μl. The mixture was cycle-sequenced starting with 3 min denaturation at 96°C, followed by 25 cycles of denaturation (96°C, 30 s), annealing (50°C, 15 s) and extension (60°C, 4 min). Thermocycle products were precipitated with 75 mM sodium acetate in ethanol, and washed with 70% (v/v) ethanol. The pellets were dried in vacuo for 3 min, and dissolved into 15 μl template-suppression reagent (Applied Biosystems). Prior to sequencing on an Applied Biosystems sequencer model 310, these samples were heated at 96°C for 2 min.

2.4. Measurement of $[Ca^{2+}]_i$ in single cells

Cells were immobilised on round glass coverslips with various coatings. Stem cells, cultured for 1 day, were adhered to coverslips coated with anti-CD34 antibody (Dako), while immature and mature megakaryocytes were adhered to coverslips coated with

Table 1
Primers used for PCR of Trp cDNAs

Trp		Primer sequence	Position	T_a [a] (°C)
1/1A	1F	5′-GAACATAAAT TGCGTAGATG-3′	622–641	52–56
	1R	5′-CGATGAGCAG CTAAAATGAC AG-3′	780–801	
1/1A	2F	5′-AACTTGGATA TACTGCAGC-3′	683–701	50–54
	2R	5′-AGAGCATTGT AAGAATTTC-3′	812–830	
3	3F	5′-TGACTTCCGT TGTGCTCAAA TATG-3′	2054–2077	58–60
	3R	5′-CCTTCTGAAG TCTTCTCCTT CTGC-3′	2348–2371	
4	4F	5′-CTGCAAATAT CTCTGGGAAG A-3′	1744–1764	46–50
	4R	5′-GCTTTGTTCG TGCAAATTTC C-3′	2135–2155	
5	5F	5′-CTGATTGCGG AAGCACTCTT CGCA-3′	2344–2367	48–54
	5R	5′-CCCTTGCAGT TGTTAGGCTC A-3′	2562–2582	
6	6F	5′-GACATCTTCA AGTTCATGGT CATA-3′	1914–1937	48–52
	6R	5′-ATCAGCGTCA TCCTCAATTT C-3′	2214–2234	
GAPDH	7F	5′-ACCACAGTCC ATGCCATCAC-3′	–	48–60
	7R	5′-TCCACCACCCTGTTGCTGTA-3′	–	

Nucleotide positions refer to those of cDNA clones Z73903 of Trp1A, U47050 of Trp3, AF175406 of Trp4, AF054568 for Trp5, and AF080394 for Trp6.

[a] Annealing temperature range.

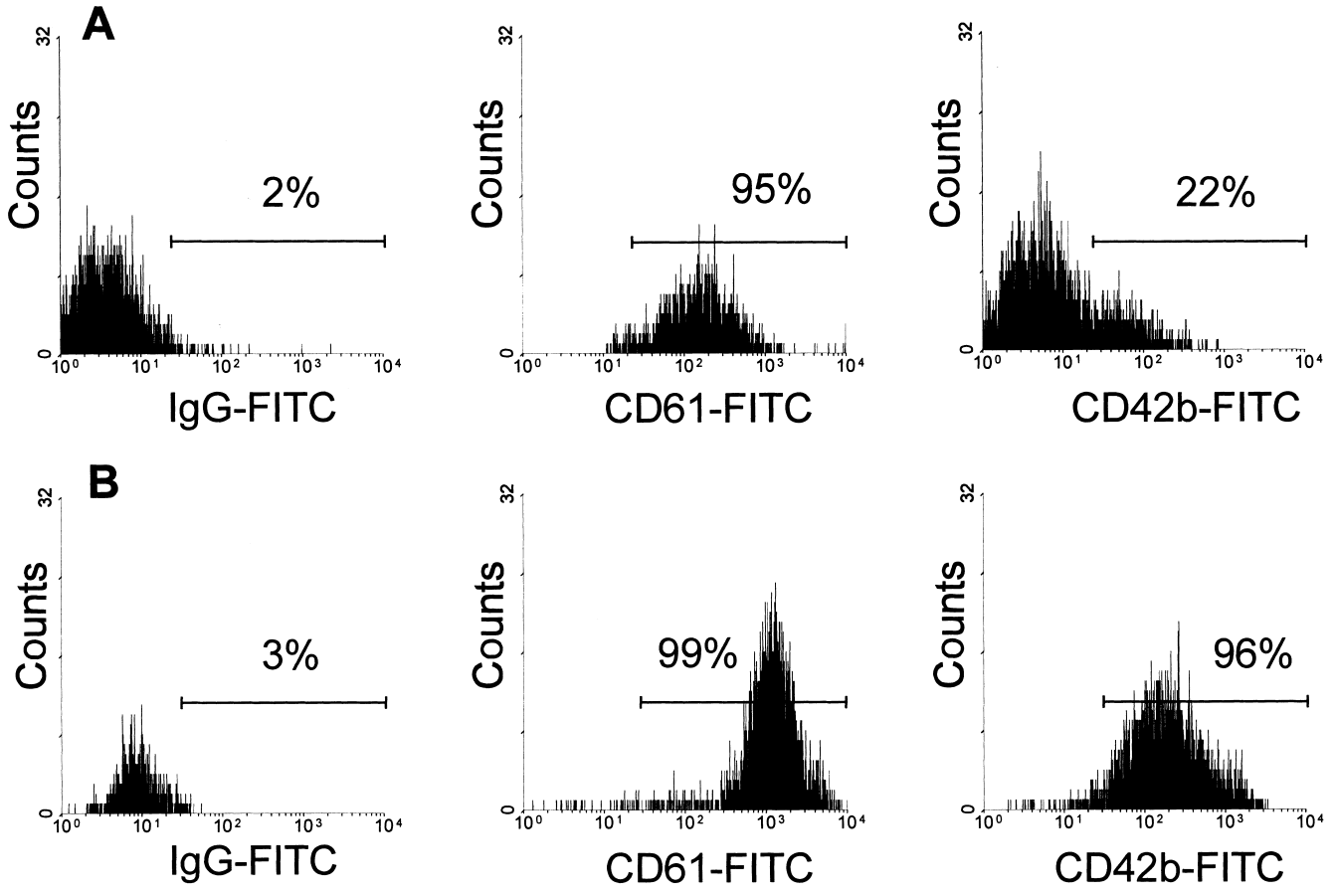

Fig. 1. Maturation stages of cells in megakaryocytic lineage. Culturing of $CD34^+$ stem cells resulted in immature megakaryocytes ($CD61^+/CD42b^{low}$) (A) and mature megakaryocytes ($CD61^+/CD42b^{high}$) (B), which were purified as described in Section 2. Plots are given of flow cytometric analyses using FITC-labelled IgG (left, control), CD61 (middle) or CD42b (right). Percentages are given of florescence counts representing FITC-labelled cells. Data are from a representative experiment.

anti-CD61 antibody. Monocytes were adhered to coverslips coated with antibody against CD14. Adhesion was during 15 min, after which the cells were incubated with 0.5 μM Fura-2 acetoxymethyl ester in HEPES buffer, pH 7.4 (145 mM NaCl, 10 mM HEPES, 5 mM KCl, 5 mM glucose, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% (w/v) bovine serum albumin). These procedures did not result in cell activation, as concluded from the continuously low levels of $[Ca^{2+}]_i$. Platelets were loaded with Fura-2, and bound to fibrinogen-coated coverslips, as described before [34]. Glass coverslips with cells were mounted in an incubation chamber laying on the stage of an inverted microscope. Changes in cytosolic $[Ca^{2+}]_i$ were recorded in individual cells by digital fluorescence microscopic imaging analysis using an intensified, charge-coupled device camera and Quanticell 750 hard- and software (Visitech, Sunderland, UK), following earlier described procedures [38]. The cells were incubated for 5 min in nominally Ca^{2+}-free HEPES buffer, pH 7.4 (145 mM NaCl, 10 mM HEPES, 5 mM KCl, 5 mM glucose and 2 mM $MgCl_2$). Thapsigargin, thrombin and/or $CaCl_2$ were added, while recording fluorescence images (background-subtracted, ratioed images of fluorescence at 340 and 380 nm excitation).

3. Results

3.1. Differentiation stages of thrombopoietin-cultured stem cells

Prolonged culturing of purified $CD34^+$ human

stem cells with thrombopoietin resulted in a gradual differentiation of the cells along the megakaryocytic lineage. By varying the culture time and by subsequent fluorescence-activated cell sorting, homogeneous populations of cells of well-defined stages of maturation could be obtained. Culturing for 1 day gave a population of primary stem cells, indexed as $CD34^+/CD61^-/CD42b^-$ (>98%). After 5 and 10 days of culturing and cell sorting, highly purified populations of $CD34^-$ immature megakaryocytes ($CD61^+/CD42b^{low}$) and mature megakaryocytes ($CD61^+/CD42b^{high}$) were obtained, respectively (Fig. 1). By selection of the 5-day grown cultures for $CD14^+$ cells, a group of cells with monocytic characteristics was obtained. The cells were identified by flow cytometry as immature monocytes, i.e., $CD14^+/CD61^-/CD42b^-$.

3.2. Species of Trp mRNA in megakaryocytes, monocytes and platelets

Human stem cells and also immature and mature megakaryocytes were analysed on mRNA of Trp isoforms. As a comparison, the collected immature monocytes were used as well as mature monocytes ($CD14^+$), which were freshly purified from peripheral blood. In addition, mRNA was characterised from the megakaryoblastic cell line Dami, known as an immortalised cell model of immature megakaryocytes [31], and from the more 'mature' megakaryo-

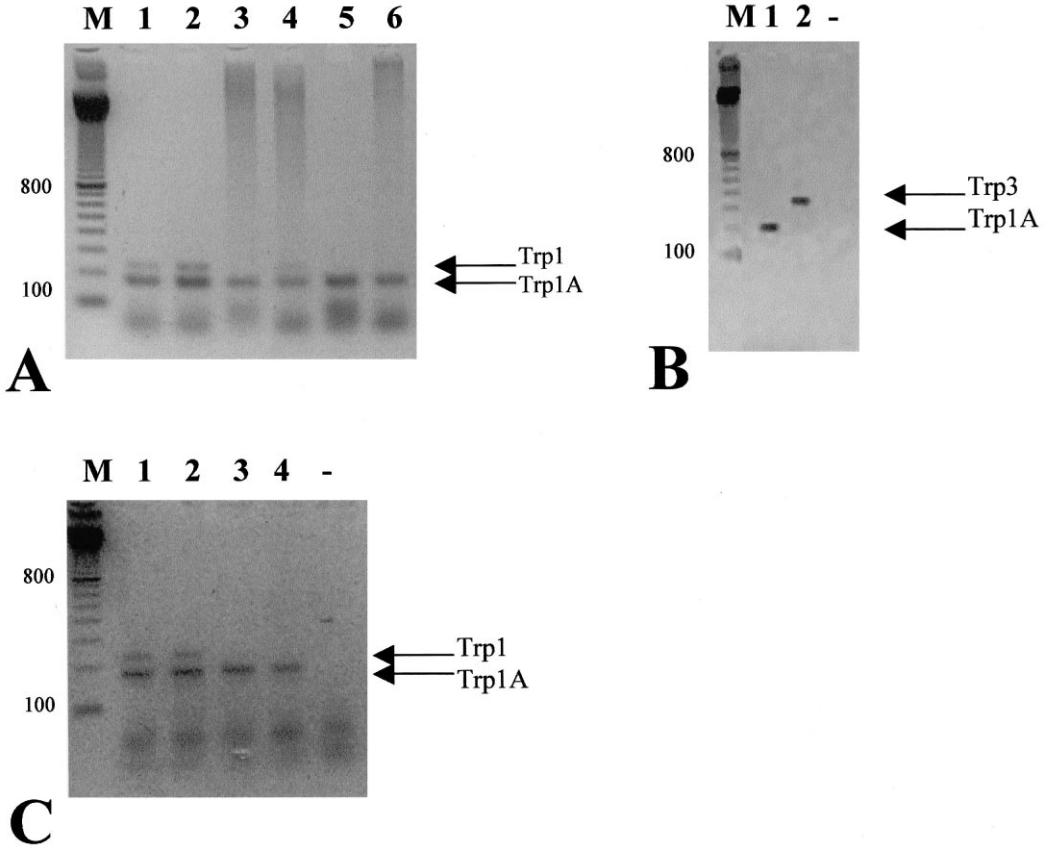

Fig. 2. Detection of Trp1 and Trp3 cDNA from different cell types. (A) cDNA from differentiating megakaryocytes and monocytes using Trp1/1A primer set 1F/1R. Lanes 1 and 2, immature and mature megakaryocytes, respectively (expected for Trp1 and Trp1A); lanes 3–6, stem cells, immature monocytes, mature monocytes and Dami cells, respectively (products of 180 bp). (B) cDNA from Dami cells using Trp1/1A primer set 1F/1R (lane 1, 180 bp) or Trp3 primer set 3F/3R (lane 2, 318 bp). (C) cDNA from platelets and DAMI cells using Trp1/1A primer set 1F/1R. Lanes 1 and 2, two platelet isolations (two bands); lanes 3 and 4, Dami cells (one band). Lanes 'M' represent 100–800 bp markers, and lanes '-' are cell-free controls. Results are representative of three or more independent experiments.

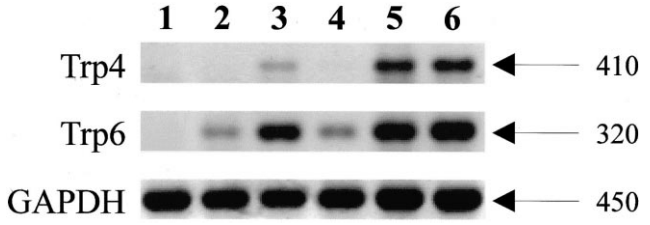

Fig. 3. Detection of Trp 4 and 6 cDNA from different cell types. cDNA from stem cells (lane 1), immature and mature megakaryocytes (lanes 2 and 3, respectively), platelets (lane 4), and Dami and CHRF-288 cells (lanes 5 and 6) using primer sets 4F/4R (upper panel), 6F/6R (middle panel) and GAPDH 7F/7R (lower panel).

cytic cell line CHRF-288 [32]. From crude lysates of these cells, mRNA was extracted using oligo(dT)$_{25}$-coated paramagnetic beads. Reverse transcriptase reaction resulted in solid-phase cDNA being covalently linked to the beads. This cDNA was subjected to PCR using primer sets specific for Trp1, 3, 4, 5 or 6 (Table 1). The primers for Trp1 were selected as to detect also the spliced variant, Trp1A. When using the latter primer set, PCR of preparations from all cell types gave a reaction product of 180 bp, indicative of the presence of Trp1A (Fig. 2A). Only cDNA derived from immature and mature megakaryocytes gave a clear, second product of 250 bp, pointing to the unspliced Trp1 variant. This band was only faint with cDNA from immature monocytes, and was absent with cDNA from mature monocytes or Dami cells. It though appeared together with Trp1A in the more mature CHRF-288 cells (not shown). Analysis for Trp2 was not performed because the expression of this form is limited to the vomeronasal organ [26]. When testing with Trp3 primers, only the Dami cells gave a positive result of 320 bp (Fig. 2B).

When using primers for Trp4, a PCR product of expected size (410 bp) was absent in stem cells and immature megakaryocytes, but it appeared in mature megakaryocytes and Dami and CHRF-288 cells (Fig. 3). Expression products of trp5 were identified in none of these cell types (data not shown). In comparison, Trp6 expression products of 320 bp were well detectable in (im)mature megakaryocytes and both cell lines, though they were not found in the stem cells (Fig. 3).

To detect transcripts of Trp isoforms in platelets, we prepared essentially leukocyte-free suspensions of human platelets (CD61^{+}/CD42b^{high}). By repeated centrifugation steps (see Section 2), a final preparation was obtained, which contained less than one leukocyte per 10^{8} platelets. This preparation was considered to be pure enough to specifically amplify the small amounts of mRNA in platelets (see below). In the presence of Trp1/1A primers, PCR of the platelet-derived cDNA resulted in bands of 250 and 180 bp, matching the sizes of the Trp1 and Trp1A fragments, respectively (Fig. 2C). For Dami cells, used as a control, again only the 180 bp product (Trp1A) was detected. With neither Trp3 nor Trp5 primers, PCR of platelet-derived cDNA gave polynucleotide products (data not shown), but primers specific for Trp4 (faint) or Trp6 (strong) gave bands of expected sizes (Fig. 3). To check for possible contamination with leukocyte mRNA material, the platelet-derived cDNA was also subjected to PCR with primers specific for the leukocyte marker, HLADQb [39]. No products were found (data not shown), suggesting that the fragments amplified from these preparations were indeed of platelet origin. Flow cytometry indicated that a significant subpopulation of the immature monocytes contained bound platelets, which could not be removed by EDTA treatment (not shown). The weak Trp1 band found with these cells might thus originate from platelet contamination.

3.3. Sequence analysis of Trp1 and Trp1A products

To confirm the identity of the amplified Trp1/1A PCR products from (im)mature megakaryocytes and platelets, restriction analysis was performed. PstI was chosen as a suitable restriction enzyme, because the Trp1-derived cDNA has two *Pst*I sites in the multiplied sequence, whereas that of Trp1A has only one site. Using the primer set 1F/2R (Table 1) and cDNA from immature megakaryocytes (Fig. 4A) or platelets (not shown), PCR yielded products of 310 and 210 bp, as expected for Trp1 and Trp1A sequences, respectively (Fig. 4A). Subsequent *Pst*I digestion showed the restriction patterns that were expected of Trp1 (202, 78 and 31 bp) and of Trp1A (78 and 128 bp).

The results of the restriction analysis were confirmed by a nested PCR method. For a first round of PCR, again the Trp1/1A outside primers 1F/2R were used (products of 210 and 310 bp). A second

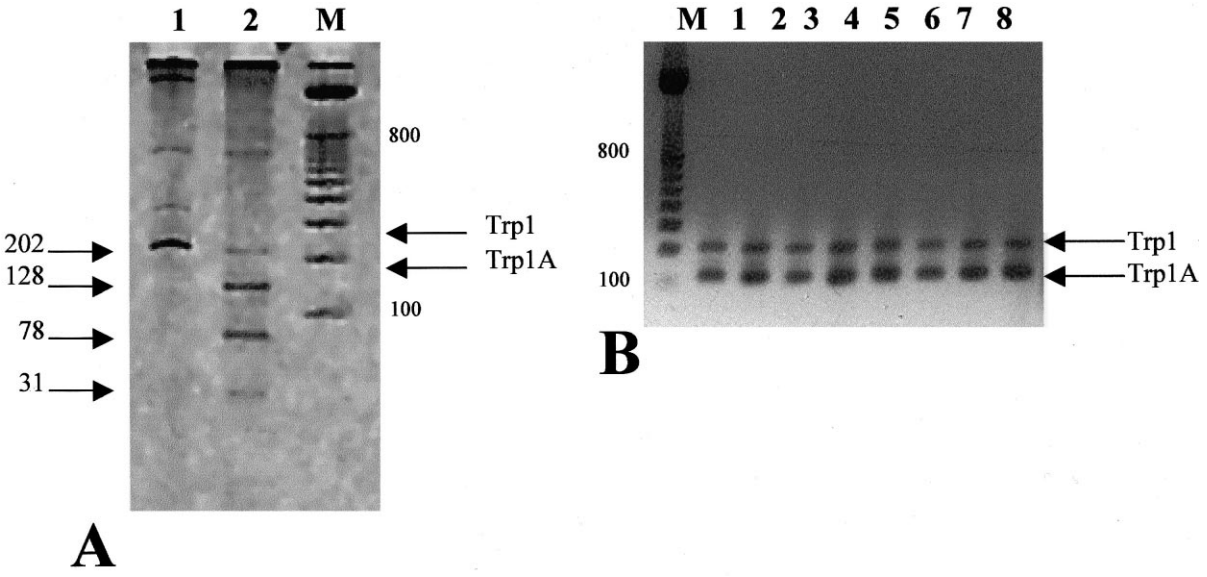

Fig. 4. Analysis of Trp1 and Trp1A cDNA products from immature megakaryocytes. (A) *Pst*I restriction analysis of Trp1/1A cDNA. Lane 1, Trp1/1A PCR products obtained from immature megakaryocytes with primer set 1F/2R (311 and 209 bp, expected for Trp1 and Trp1A, respectively); lane 2, *Pst*I analysis of the PCR products. Note the 202, 78 and 31 bp restriction bands indicative of Trp1, and the 128 and 78 bp bands indicative of Trp1A. (B) Nested PCR analysis of Trp1/1A cDNA. Trp1/1A cDNA products were generated with (outside) primer set 1F/2R (311 and 209 bp), after which a second PCR was performed with (inside) primers 2F and 1R. Lanes 1–8 are results from multiple assays, showing nested PCR products of 221 bp (Trp1) and 119 bp (Trp1A). Lanes 'M' represent 100–800 bp markers.

PCR round was then run with nested 2F/1R primers, which appeared to amplify products of 220 and 120 bp (Fig. 4B), matching the sizes of the predicted Trp1 and Trp1A fragments, respectively.

Finally, using material from immature megakaryocytes and Dami cells, the identity of the obtained Trp1 and Trp1A cDNA was confirmed by direct sequencing of the PCR products (primers 1F/1R). The PCR products were purified from gel and thermocycled with fluorescent dideoxynucleotides prior to sequencing. The obtained sequences matched those of the described cDNA clones (GenBank numbers) U731110 (Trp1) and Z73903 (Trp1A) [15,19]. Together, these results indicate that the multiplied PCR products are indeed derived from the two variant forms of Trp1 and Trp1A cDNA.

3.4. Store-regulated calcium entry in various cells

Considering that the differential expression of Trp mRNA forms in the megakaryocyte and monocyte lineages extends to the molecular make-up of Trp proteins (see below), this difference may have consequences for the store-regulated Ca^{2+} entry through Trp channels. We therefore investigated this process by loading the various cells with the Ca^{2+} probe Fura-2, and determining the increases in $[Ca^{2+}]_i$ after treatment of the cells with the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (2.5 μM), and subsequent application of $CaCl_2$ (1 mM). According to this standard protocol of measuring SOCE, thapsigargin causes depletion of the Ca^{2+} stores in the endoplasmic reticulum, which triggers the influx of added extracellular Ca^{2+}. The results with various cells show that thapsigargin evoked a similar, slow increase in $[Ca^{2+}]_i$ in $CD34^+$ stem cells and (im)mature megakaryocytes, whereas the subsequent Ca^{2+} signal with $CaCl_2$ was much higher in case of the megakaryocytes (Fig. 5); e.g., in immature megakaryocytes it was 3.4 ± 0.6 (mean ± S.D., $n = 3$) times higher than in stem cells. With platelets, thapsigargin evoked a transient, somewhat higher increase in $[Ca^{2+}]_i$, probably caused by a potentiating effect of thromboxane formation on store depletion [2], after which the $[Ca^{2+}]_i$ decreased to a slightly elevated level. The Ca^{2+} influx by subsequent $CaCl_2$ addition was similarly high as in megakaryocytes (Fig. 5). Applying this protocol to Fura-2-loaded

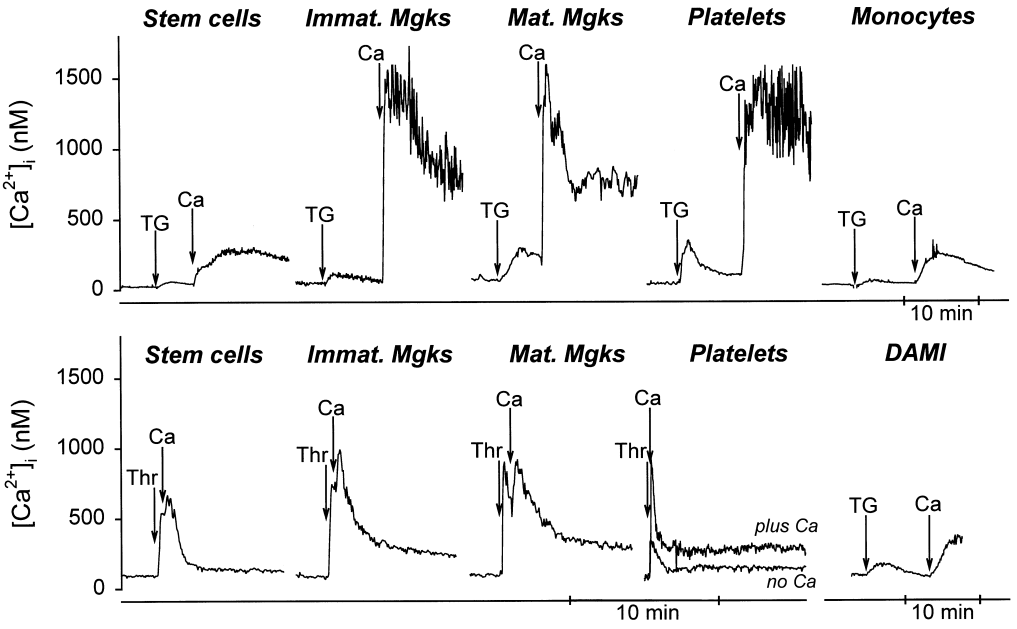

Fig. 5. Changes in store-regulated Ca^{2+} entry upon stem cell differentiation. Cells were immobilised on coverslips and loaded with Fura-2, as described in Section 2. Changes in Fura-2 fluorescence ratio were determined in single cells by microscopic video imaging techniques. The cells on coverslip were incubated in nominally Ca^{2+}-free medium and stimulated with 2.5 μM thapsigargin (TG) or 10 nM thrombin (Thr), followed by 1 mM $CaCl_2$ (Ca), as indicated. Traces are results obtained with stem cells, immature megakaryocytes (Immat. Mgks), mature megakaryocytes (Mat. Mgks), platelets, mature monocytes and Dami cells. In the case of platelets, a control curve is given where $CaCl_2$ was not added. Averaged responses are given from 15–20 cells in a representative experiment ($n > 3$).

monocytes and Dami cells resulted in a small thapsigargin-induced increase in $[Ca^{2+}]_i$, which was followed this time by a small Ca^{2+} influx signal upon $CaCl_2$ addition. Thus, with a comparable degree of Ca^{2+}-store depletion by thapsigargin, the influx of Ca^{2+} was much higher in megakaryocytes and platelets than in the other investigated cell types.

For the primary cells, we verified that the high Ca^{2+} influx signal was also present after stimulation with a Ca^{2+}-mobilising receptor agonist like thrombin. Pilot experiments using the SOCE protocol indicated that $CaCl_2$ needed to be added shortly after appearance of the thrombin-evoked $[Ca^{2+}]_i$ peak for optimal detection of the influx, probably because of rapid refilling of the Ca^{2+} stores with this agonist. When $CaCl_2$ was not added, thrombin (10 nM) induced a short $[Ca^{2+}]_i$ transient in stem cells, (im)mature megakaryocytes and platelets which was mostly completed within 3 min of activation (shown for platelets in Fig. 5). When 1 mM $CaCl_2$ was added shortly after thrombin, the Ca^{2+} signal of the stem cells remained short in duration, while that of the (im)mature megakaryocytes became much more prolonged. Similarly in platelets, $CaCl_2$ addition resulted in a prolonged elevated $[Ca^{2+}]_i$ level (Fig. 5). As we have described elsewhere [4], in individual platelets this long-term elevation appeared to be composed of multiple $[Ca^{2+}]_i$ spikes. Thus, in thrombin-stimulated megakaryocytes and platelets but not in stem cells, a prolonged Ca^{2+} signal was detectable under conditions permitting Ca^{2+} influx.

4. Discussion

In this study, we present evidence for a variation in Trp mRNA expression in human stem cells that differentiate to megakaryocytes/platelets or monocytes. In stem cells ($CD34^+$), we detected only mRNA of the spliced variant Trp1A, whereas that of unspliced Trp1 or Trp3–6 was absent. Immature $CD14^+$ monocytes (derived from the stem cells) and mature monocytes (isolated from peripheral blood) showed a similar expression of only the spliced

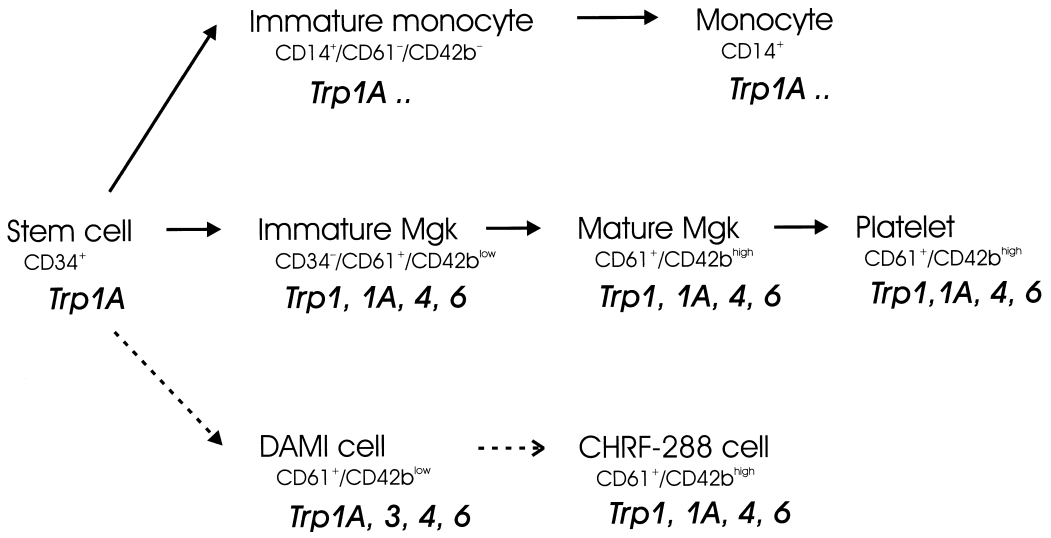

Fig. 6. Overview of detected Trp transcripts in human stem cells, megakaryocytes, monocytes, platelets and related cell lines.

Trp1A form. The PCR of the cDNA material from immature monocytes gave a very faint band of the size of Trp1. This may, however, have resulted from low contamination of the monocyte preparation with $CD61^+$ cells (approximately 2–4%). Such a band was never detected in cDNA from the mature monocytes.

The immature and mature megakaryocytes (originating from thrombopoietin-cultured stem cells) and also platelets (well-purified from blood) contained mRNA of both the unspliced Trp1 and the spliced Trp1A isoforms. In addition, these cells expressed Trp4 and 6, while Trp3 and 5 transcripts could not be detected (see Fig. 6). A somewhat different pattern of Trp transcripts was obtained with the immortalised, megakaryoblastic cell-line, Dami, i.e., mRNA was present from Trp1A, 4 and 6 as Trp3, but not of the unspliced Trp1 form. In contrast, the more 'mature' megakaryoblastic CHRF-288 cells (characterised by high surface levels of CD61 and CD42b) contained the same Trp expression pattern as (mature) megakaryocytes and platelets. Accordingly, as summarised in Fig. 6, the unspliced Trp1 variant was found to be present in all cell types expressing CD61 and CD42b antigens, except for the Dami cells. Using different primer sets, other authors have also described the presence of Trp1 (Trp1A?) and Trp3 transcripts in the Dami cells [30]. Thus, although the Dami cells show similarities with immature megakaryocytes [41], their Trp pattern does not appear to reflect the megakaryoblastic origin of these cells. Note that Dami cells also differ from human platelets in the expression pattern of plasma membrane Ca^{2+}-ATPases [42].

The simultaneous presence of differently spliced isoforms of Trp1 mRNA, as we have observed these in platelets and megakaryocytes, is not a new finding, since this is also detected in bovine aortic endothelial cells [24] and a mouse insuloma cell line [43]. The Trp1A spliced variant is cloned and is deposited under the numbers X89066 and Z73903 (GenBank). Its deduced amino acid composition typically differs from that of Trp1 in a shorter N-terminal cytoplasmic part [15,19]. Transfection studies with model cells have shown that both the longer and shorter forms of the Trp1 protein can carry a cation current that is elicited by store depletion and is permeable for Ca^{2+} and monovalent cations like Na^+ [16–18]. Although the properties of these protein forms have not yet been compared quantitatively, it is relevant to note that a truncation of Trp1 at the C-terminal cytoplasmic tail alters its capacity to SOCE in HSG cells [44]. This implies that cytoplasmic parts of Trp1 can regulate the channel activity.

Using fluorescent imaging techniques, we found that human stem cells, Dami cells and monocytes are relatively low in capacity of Ca^{2+} entry after store depletion when compared to (early) megakaryocytes and platelets (Fig. 5). This first means that the capability of SOCE is present in all these cells, including the undifferentiated stem cells. This is com-

patible with detection of the Trp1A gene transcript in all cell types. Second, the high Ca^{2+} influx in megakaryocytes and platelets occurred after a similar degree of Ca^{2+} store mobilisation by thapsigargin, indicating that it was unlikely to be caused by a different degree of store depletion. Experiments with the receptor agonist thrombin also pointed to a more prolonged Ca^{2+} influx-dependent Ca^{2+} signal in (im)mature megakaryocytes and platelets. Differentiation towards to megakaryocytic/platelet lineage thus appears to be accompanied by increased SOCE.

The present data suggest involvement of additionally expressed Trp channels in the increased store-regulated Ca^{2+} entry upon differentiation. Involvement of Trp forms agrees well with the early finding of Avdonin et al. [45] that much of the Ca^{2+} influx in platelets occurs via store-regulated, non-selective cation channels. This points to the Trp1, 4 and 6 proteins, providing non-selective cation channels, rather than the newly identified Ca^{2+}-release activated channel, CaT1, which is highly Ca^{2+} selective [46]. Knowing that the Trp4- and 6-expressing Dami cells have a relatively low SOCE (Fig. 5), it might be deduced that especially the Trp1 and 1A forms are responsible for the high Ca^{2+} entry in megakaryocytes and platelets. Different Trp isoforms can assemble into heteromultimeric channels [23,40], which makes it not unlikely that co-expression with Trp1 and/or Trp1A upon maturation changes the overall SOCE activity in the differentiated stem cells. The extra deduced amino acid stretch in the long Trp1 variant contains consensus phosphorylation sites for the protein kinases A, C, and G (D. Molin, unpublished results), suggesting that the protein is subjected to phosphorylation regulation. Both in (rat) platelets [4,8] and megakaryocytes [47], protein kinase A and G stimulation indeed modulate the Ca^{2+} influx signal, although at least in part indirectly by reducing the Ca^{2+} store depletion. Calcium entry in platelets is also known to be regulated by protein kinase C. While the newest evidence suggests that this influx pathway is independent of Ca^{2+}-store depletion [48], it may still be mediated by (diacylglycerol-sensitive?) Trp channels. Clearly, more detailed expression studies of Trp1 and 1A and perhaps specific antagonists are required to confirm these deductions.

It has recently been established that human platelets contain significant amounts of Trp1 protein (using an antibody directed against an amino acid sequence at the C-terminal moiety), and that this protein couples to the platelet $InsP_3$ receptors upon Ca^{2+} store depletion [49]. The present qualitative mRNA analysis suggests, but does not unequivocally demonstrate, that the unspliced Trp1 protein participates in this coupling. In addition, the apparent absence of Trp3 mRNA in platelets and megakaryocytes can explain why the transient mechanism of Trp-$InsP_3$ receptor coupling in platelets differs from the more stable interaction between Trp(3) and $InsP_3$ receptors in other types of cells transfected with Trp3 channels [49,50].

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